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A comparative study of different strategies for removal of endotoxins from bacteriophage preparations

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Abstract

Bacterial endotoxins have high immunogenicity. Phage biology studies as well as therapeutic phage applications necessitate highly purified phage particles. In this study, we compared combinations of seven different endotoxin removal strategies and validated their endotoxin removal efficacy for five different phages (*i.e.* four *Pseudomonas aeruginosa* phages and one *Staphylococcus aureus* phage). These purification strategies included Endotrap HD column purification and/or CsCl density centrifugation in combination with Endotrap purification, followed by organic solvent (1-octanol), detergent (Triton X-100), enzymatic inactivation of the endotoxin using alkaline phosphatase and CIM monolytic anion exchange chromatography. We show that CsCl density purification of the *P. aeruginosa* phages, at an initial concentration of 10^{12} - 10^{13} pfu/ml, led to the strongest reduction of endotoxins, with

an endotoxin removal efficacy of up to 99 %, whereas additional purification methods did not result in a complete removal of endotoxins from the phage preparations and only yielded an additional endotoxin removal efficacy of 23 to 99 %, sometimes accompanied with strong losses in phage titer.

Keywords

Endotoxins, lipopolysaccharides (LPS), Bacteriophages, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Endotoxin removal, Endotoxin quantification.

1. Introduction

The purification of bacteriophage particles is important for two reasons: either to investigate the phage particle on its own (*i.e.* phage biology studies) or for therapeutic application of phages, which is currently undergoing a resurgence (Adhya et al., 2014; Krystyna Dabrowska et al., 2014; Ly-Chatain, 2014; Miedzybrodzki et al., 2012; Thiel, 2004; Vandenheuvel et al., 2015). When phages are propagated on Gram-negative bacterial hosts, endotoxins or lipopolysaccharides (LPS) have to be removed from these preparations. Endotoxins are part of the Gram-negative bacterial outer membranes and play an important role in the organization and stability of the bacterial cell (Ki et al., 1994). Bacterial endotoxins are well known for their immunogenic, pro-inflammatory and pyrogenic effects (Aderem and Ulevitch, 2000). In conditions where the body is exposed to endotoxins excessively or systemically, a systemic inflammatory reaction can occur, leading to multiple pathophysiological effects such as endotoxin shock, tissue injury and death (Anspach, 2001; Erridge et al., 2002; Ogikubo et al., 2004). Therefore, when phages are prepared for therapeutic purposes, it is crucial that different bacterial contaminants are removed which affect the efficacy and safety of the administration during phage therapy. The maximal level of endotoxins for intravenous applications of pharmaceutical and biological products is set at 5 endotoxin units (EU), i.e. 500 pg of endotoxins^{10}, per kg of body weight per hour (Daneshian et al., 2006). Additionally, bacterial endotoxins may also interfere with phage biology studies, especially when trying to establish the interaction of phages with the immune system.

Several strategies have been described for the removal of endotoxins from phage preparations. Here we compared different endotoxin removal strategies for the removal of endotoxins from five phages, *i.e.* four Gram-negative *Pseudomonas aeruginosa* phages and one Gram-positive *Staphylococcus aureus* phage (Table 1). The *S. aureus* phage forms a negative control for the endotoxin determination assay, as this phage is grown on a Gram-positive host which produces no endotoxins. Strategies were compared, taking into account the efficacy in removing endotoxins in relation to their effect on the phage titer yield.

2. Results and discussion

In this study, we evaluated the endotoxin removal efficacy of seven purification strategies (Figure 1). To determine which strategy has the best endotoxin removal capacity, in combination with the minimal amount of phage loss, we calculated the 'endotoxin removal efficacy', defined as the ratio of the endotoxin units (EU) per plaque forming unit (pfu) multiplied by the phage recovery of the purified sample and the original sample subtracted from one (Table 2). The endotoxin quantification by Endozyme was validated by endotoxin quantification by means of Endosafe-PTS, for a selected number of samples. Both detection methods gave similar results within the same order of magnitude (Table S1)

The endotoxin removal strategies include either (1) Endotrap HD column purification alone (Merabishvili et al., 2009) (ϕET), or (2) CsCl density gradient ultracentrifugation alone (Lavigne et al., 2009) (ϕC) or (3) followed with Endotrap HD purification (ϕCET),

and ϕET or ϕCET followed by either (4) organic solvent (1-octanol; Szermer-Olearnik and Boratyński, 2015) treatment (OS), (5) detergent Triton X-100; Marcus and Prusky, 1987; Petsch and Anspach, 2000) treatment (TX), (6) enzymatic inactivation of the endotoxin using alkaline phosphatase (Bentala et al., 2002) (AP) or (7) anion-exchange chromatography (CIM DEAE disk column (CIM); Adriaenssens et al., 2012). We opted for these combined strategies, to compare the efficacy of purifying raw phage lysates (ϕET) versus CsCl-purified phages (ϕCET). As expected, phage ISP preparations from a Gram-positive host did not show any detectable endotoxin levels before or after any of the purification strategies (Table S1). The four *P. aeruginosa* phages contained between 326,000 and 7,465,000 EU/ml (Table S1). This concentration was measured after purification of phage lysates with Endotrap HD and therefore may initially be higher. However, it is possible that these values correspond largely to the initial order of magnitude of the endotoxins present in the crude phage preparations, because the removal efficacy of the Endotrap HD purification, starting from the CsCl purified phages, was only between 63.09 – 94.56 % (Table 2). Cooper *et al*. (2014) showed that crude *P. aeruginosa* phage preparations contained more than 10^5 EU/ml, which was in correspondence with our quantification of preparations that were purified only once with Endotrap HD. Dufour *et al*. (2016) recently described that three to five consecutive rounds of Endotrap HD-based endotoxin removal, starting from CsCl purified phages, led to a further reduction of the endotoxin concentration to below 0.5 EU/ml, but this is a time-consuming strategy with a variable and phage-dependent outcome.

2.1. Organic Solvent (OS)

The organic solvent extraction was described by Szermer-Olearnik and Boratyński (2015) and is based on the principle that endotoxins partition favorably in the organic phase, while the molecules of interest (in our case phages) are retained in the aqueous phase. Our results show that the organic solvent strategy has an endotoxin removal efficacy between 63.83 and 99.98 % for ϕET and between 23.40 and 63.36 % for the ϕCET samples. The only exception is *P. aeruginosa* phage LUZ19 where there was an enrichment of endotoxins after the 1-octanol treatment, *i.e.* an endotoxin removal efficacy of -16.55 %. This enrichment could be explained by a potential release of endotoxins bound to the phage particle, which when bound are not detectable in the endotoxin detection assay.

The organic solvent strategy led to strongest reduction of endotoxins in the *P. aeruginosa* phage PNM ϕET and phage LUZ19 ϕET preparations, with an endotoxin removal efficacy of 99.98 and 99.68 % respectively. This was only accompanied with a reduction of the *P. aeruginosa* phage PNM with two orders of magnitude. For both phages, a strong reduction in phage titer of up to five orders of magnitude difference was observed.

2.2. Detergent: Triton X-100

The endotoxin removal strategy using detergents such as Triton X-100 has been well-established. According to Petsch and Anspach (2000), endotoxins can be removed using a two-phase extraction, employing detergents such as those of the Triton series.

An endotoxin removal efficacy of up to 93.21 % and 99.96 % could be observed for the ϕET and ϕCET phage samples, respectively (Table 2). The use of activated charcoal for the removal of Triton X-100 led to a reduction in the endotoxin concentration after three consecutive rounds. Around 6.29 – 7.14 % endotoxins remained for the ϕET preparations and between 0.31 – 43.6 % endotoxins remained for the ϕCET preparations. However, the activated carbon was not able to remove all of the Triton X-100. This became apparent when the samples became viscous when stored at 4 °C for prolonged time. The phage recovery for the ϕET was between 0.07 – 17.20 % and for the ϕCET it was between 1.80 – 52.22 % (Table 3), indicating that the Triton X-100 strategy has an effect on the phage activity.

2.3. Enzymatic inactivation of endotoxins: alkaline phosphatase

An alternative method for the removal of endotoxins is enzymatic inactivation of endotoxins by the removal of the phosphate group of the lipid A fraction by means of alkaline phosphatase (Bentala et al., 2002). Consequently, the treated endotoxin becomes immunologically inactive and should not be detected in a classical Limulus Amebocyte Lysate (LAL) assay or by recombinant factor C (rFC) assays as used in this study. However, the treatment of the ϕET and ϕCET phage preparations with alkaline phosphatase had very low endotoxin removal efficacies (*i.e.* lower then 20 %; Table 2) and did not lead to a reduction in the endotoxin concentration (Table S1). In addition this method had a negative impact on the number of infectious phage, dropping 2 – 4 orders of magnitude (Table 3).

Although this strategy has been described for the inactivation of purified endotoxins, when it was applied on phage samples there was no reduction in the endotoxin concentration. This might indicate that the phages either inhibit the enzymatic activity of the alkaline phosphatase or that the phages, through the binding of the endotoxins, hide the phosphate groups on the lipid A part. Consequently, this can result in the endotoxins not being completely inactivated by the enzyme while still being detectable in the endotoxin detection assay.

2.4. Anion-exchange purification: CIM DEAE disks

The final strategy that was evaluated was the anion-exchange purification using CIM DEAE columns. This technique was previously described for its application in the purification and concentration of phages (Adriaenssens et al., 2012), but the authors did not evaluate the endotoxin removal potential. In this strategy, the phages are retained on the column through ionic interaction. This purification protocol was only performed for the ϕET preparations of *P. aeruginosa* phages PNM and LUZ19. Although the two phages that were purified by this method (*i.e. P. aeruginosa* phage PNM and LUZ19) have a high sequence similarity, the endotoxin removal efficacy varied between these two phages (*i.e.* 98.15 and 40.39 %, respectively; Table 2). A strong reduction in phage titers from 10^{13} to 10^{11} pfu/ml for both the *P. aeruginosa* phages PNM and LUZ19 was observed (Table 3). The limited reduction in endotoxin concentration after the anion-exchange purification could be explained by the fact that endotoxins also have the ability to interact with anion-exchange columns.

According to Hou and Zaniewski (1990), the capacity of endotoxin removal by anion-exchange matrices through charge interaction depends on the number of available positively charged groups existing in the matrices. They observed a maximum adsorption of endotoxins at pH 6.8 when DEAE columns were used, and at pH 8.0 when QA columns were used. They also found that the endotoxin adsorption was found to be unaffected at up to 0.2 M salt concentration (Hou and Zaniewski, 1990). We found that both phages were retained at the column at a pH of 7.5 and eluted at a NaCl concentration above 0.6 M. Therefore, the removal of endotoxins without loosening the phages from the column is cumbersome. Hence, the endotoxins co-elute with the phages minimizing the ability to obtain endotoxin free phage preparations. The reduction in phage titers results from the phage binding capacity of the columns, which is phage-dependent.

3. Conclusions

Starting from between 2.5 x 10^{12} and 8 x 10^{13} pfu/ml of 4 different *P. aeruginosa* phages (Table 3), contaminated by 31,020 and 7,465,000 EU/ml (Table S1), we found that CsCl density gradient ultracentrifugation established an endotoxin removal efficacy between 18.42 and 99.68 % (Table 2) while reducing the number of phages with maximum two orders of magnitude (Table 3). Further endotoxin removal of these CsCl preparations with endotrap, OS, TX, AP or CIM did reduce endotoxins further with a maximum endotoxin removal efficacy of 99.9 %, whereas several of

these additional treatments were detrimental for the phage titer, which was even reduced to 0.0004 % for the *P. aeruginosa* phage PNM ϕCET OS treated sample.

From our comparative study, it becomes clear that it is hard to achieve complete removal (*i.e.* ≥ 99.99 %) of endotoxins from a phage sample. For therapeutic purposes, only 5 EU/ml/kg/h can be present in the samples for intravenous applications (Daneshian et al., 2006). We clearly find that the removal of large volumes of endotoxins is easier than the removal of small residual endotoxins, as the endotoxin removal efficacy of the different procedures starting from the phage lysate (ϕET) preparation is much higher than those form the CsCl purified (ϕCET) preparations (Table 2). Unfortunately, we were not able to obtain an universal strategy that could be used for the removal of endotoxins from any given phage preparation. Therefore, each phage needs to be evaluated individually for the optimal strategy for the removal of endotoxins, taking into account the potential drop in phage titers. The CsCl purification (ϕC) seems to have the highest efficacy in removing endotoxins. Although this technique might not be suitable for all applications such as phage therapy where high throughput and up scaling is a must, this strategy might be important for phage biology studies in which endotoxin contamination may result in confounding effects.

Since the phage titers used for phage therapy are usually around 10^8 pfu/ml (Merabishvili et al., 2009), the phage preparations can be further diluted leading to a further drop of the endotoxin concentration (ranging from 0.0002 to 316 EU/ml for

10^8 pfu/ml phage preparations; Figure 2) and subsequent safe use. Therefore, labor intensive endotoxin removal strategies should not be necessary for therapeutic phage preparations, knowing that the dilution of high titer phage preparations would be sufficient. Unfortunately, dilution of the phage preparations is not always possible when performing phage biology studies such as evaluating the immunological properties of phages on the mammalian immune system, where high phage titers might be advised (Biswas et al., 2002; Miernikiewicz et al., 2013).

Although this study has as a limitation that no technical replicates were obtained for the different endotoxin removal strategies, our results do indicate that the endotoxin removal is phage dependent, and thus needs to be evaluated individually for each phage. To obtain a complete removal of endotoxins, a combination of strategies could be used, such as treating the phage lysate with Triton X-100 followed by a purification and concentration of the phages through means of anion-exchange (*i.e.* DEAE disks) or by using consecutive rounds of Endotrap HD endotoxin removal after CsCl density centrifugation, as suggested by Dufour *et al.* (2016).

4. Material and methods

4.1. Bacteriophage and bacteria

4.1.1. Phage propagation

Bacteriophage stocks (Table 1) were prepared using the double-agar overlay method as described in Merabishvili *et al.* (2009) (Merabishvili et al., 2009). Briefly, one ml of the phage preparation containing 10^6 plaque forming units (pfu) of bacteriophages

was mixed with 3 ml of molten (45 °C) Lysogeny Broth (LB) (Becton Dickinson, Erembodegem, Belgium) top Bacto agar (0.6 %) (Becton Dickinson) and 100 µl of the host strain suspension (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube (Falcon, Becton Dickinson). This mixture was plated onto freshly made 90 mm diameter Petri dishes (Plastiques Gosselin, Menen, Belgium), filled with a bottom layer (20 ml) of 1.5 % LB agar, and incubated aerobically at 32 °C for 16 h. Subsequently, 200 µl of chloroform was added to the lids of the Petri dishes and the inverted plates were further incubated at 4 °C for 1 h. The top layer of the double-agar layer was scraped off using a sterile Drigalski spatulum and transferred to a sterile 14 ml tube.

The harvested phages were centrifuged for 20 min at 6,000 x *g* at 4 °C. The supernatant was aspirated using a sterile 10 ml syringe (BD Plastipak, Becton Dickinson) with a 30 G sterile needle (BD Microlance 3, Becton Dickinson) and passed through a 0.22 µm membrane filter (Sartorius Stedim, Zellik, Belgium). The filtrate was subsequently centrifuged at 35,000 x *g* for one hour. The phage pellet was resuspended in 5 ml saline and stored at 4 °C overnight before determining the phage titer. Preferably, the titer of the phage lysate should be checked at least one day later according to the above described procedures. This will allow phage particles that may have clumped together during centrifugation steps to disengage (Kutter and Sulakvelidze, 2004).

4.1.2. Phage titer determination

The bacteriophage titer was determined by assaying decinormal serial dilutions (log(0) to log(-12)) of the bacteriophage suspension with the overlay method (Merabishvili et al., 2009). One ml of each dilution was mixed with 3 ml of molten (45 °C) LB 0.6 % top Bacto agar and the host strain (end concentration of 10^7 cfu/ml) in a sterile 14 ml tube. This mixture was plated in triplicate onto 90 mm diameter Petri dishes, filled with a bottom layer of 1.5 % LB agar, and incubated for 16 h at 37 °C. To determine the original bacteriophage concentration, plates with one to 100 distinguishable homogenous plaques were counted. The mean was then calculated for the triplicate plates.

4.2. Endotoxin removal strategies

All manipulations were carried out using endotoxin-free reagents. Figure 1 gives a schematic representation of the different purification strategies used.

4.2.1. CsCl purification

Phage lysates particles were further purified and concentrated by means of ultracentrifugation (104,000 x *g*, 4 °C) for 3.5 h in a CsCl (PanReac AppliChem, Darmstadt, Germany) gradient with densities of 1.33 to 1.70 g/cm^3 in a swigging bucket centrifuge as described by Lavigne et al. (2009). The resulting high-titer phage suspension (ca. 3 – 4 ml) was dialyzed with a Slide-A-lyzer Mini Dialysis device (10,000 MWCO, Thermo Scientific, Hudson, NH) three times for 30 min at 4 °C against 1 l of

saline to remove residual CsCl. The CsCl-purified phage samples (ϕC) were stored at 4 °C and the phage titer was determined on the following day.

4.2.2. Two-phase extraction

4.2.2.1. Organic solvent (OS) treatment: 1-octanol

Phage ϕET and ϕCET preparations were treated with an organic solvent (1-octanol; Sigma-Aldrich, Munich, Germany), as described by Szermer-Olearnik and Boratyński (2015). Briefly, 500 µl of the phage preparation (either phage lysate (ϕET) or CsCl purified phages (ϕCET)) was transferred to a 1.7 ml Eppendorf tube. Subsequently, $MgCl_2$ (Sigma-Aldrich) was added to a final concentration of 0.02 M. This mixture was incubated for 1 h at 4 °C. After incubation, 1-octanol (40 % v/v) was added and mixed overnight by inverting in a vertical rotator at room temperature. The mixture was incubated for 1 h at 4 °C prior to centrifugation at 4,000 x *g* for 10 min. The upper 1-octanol phase was removed and the lower aqueous phase was transferred to Slide-A-lyzer Mini Dialysis device (10,000 MWCO). Dialysis was performed against ethanol (25 %) for five subsequent rounds (one overnight incubation and four 2 h incubations). Subsequently, the samples were dialyzed against endotoxin-free saline for four rounds (one overnight and three incubations of two hours). The purified phage solution was stored overnight at 4 °C and the phage titer was determined on the following day.

4.2.2.2. Detergent treatment: Triton X-100

The Triton X-100 removal of endotoxins is based on general protocols for the removal of endotoxins from protein preparations, as described by Petsch and Anspach (2000). Above the critical micelle concentration of some detergents, endotoxins are trapped in a micellar structure by non-polar interactions of the alkyl chains of lipid A and the detergent and are consequently separated from the water phase.

Detergents of the Triton series show a miscibility gap in aqueous solution. Above a critical temperature, the so-called cloud point, micelles aggregate to droplets with very low water content, thus forming a new phase. Endotoxins remain in the detergent-rich phase. The cloud point of Triton X-114 is at 22 °C, which is advantageous when purifying proteins. It requires mixing of the endotoxin-containing protein solution in the cold (usually at 4 °C) and allows separation of the two phages at temperatures of 22 °C or above. In contrast, the cloud point for Triton X-100 is at 75 °C, which is not acceptable for most proteins or phages, because high temperatures might lead to inactivation. In the classical protocols, Triton is removed by centrifugation. This has as downside that it is impossible to remove all the Triton present in the sample. Alternatively, Triton can be removed by using activated charcoal (Marcus and Prusky, 1987), which has as an additional advantage that it is not necessary to use a two-phase system, which makes it possible to use Triton X-100 (which has, due to its higher cloud point, the advantage of being used at room temperature).

A total volume of 200 µl of the phage solution (*i.e.* ϕET or ϕCET) was transferred to a 1.7 ml Eppendorf tube. To this solution 3 % (v/v) Triton X-100 (Sigma-Aldrich) was added and incubated for 30 min at room temperature while shaking at 750 rpm. After incubation, 12 % activated carbon (Sigma-Aldrich) was added to remove the Triton X-100 (Marcus and Prusky, 1987). An additional 30 min of incubation was performed at room temperature while shaking at 750 rpm. The solution was centrifuged at maximum speed for 1 min, after which it was passed through a 0.45 µm membrane to remove residual activated carbon. All steps were repeated for an additional two rounds, i.e. a total of three Triton X-100 treatments. The purified phage solution was stored overnight at 4 °C and the phage titer was determined on the following day.

4.2.3. Enzymatic inactivation of endotoxins: alkaline phosphatase (AP)

The inactivation of endotoxins through enzymatic degradation by means of alkaline phosphatase was described by Bentala et al. (2002). Briefly, alkaline phosphatase (rSAP (1,000 U/ml;7.5 U/reaction of 300 µl), New England Biolabs, New England, MA) together with the CutSmart buffer (New England Biolabs; as described by the manufacturer) was added to the phage sample and incubated for 60 min at 37 °C. Subsequently the enzyme was inactivated by heating the solution for 5 min at 65 °C. Following endotoxin inactivation, the titer of the phage solution was determined.

4.2.4. Adsorption techniques

4.2.4.1. Endotrap HD

Endotrap HD is an affinity chromatography based strategy using bacteriophage-phage derived proteins that are fixed on the column matrix and bind endotoxins with a high affinity and specificity. According to the manufacturer, Endotrap HD is able to remove endotoxins from protein, peptides, antibodies, RNA/DNA, antigens and plant extract samples with an endotoxin removal efficiency of 99.99 %. It also is claimed to have a wide pH range (4 – 10) of activity and that high salt concentrations do not affect the endotoxin removal capacity.

The phage lysates (ϕET) or CsCl purified phage lysates (ϕCET) were further purified from endotoxins using the commercially available kit Endotrap HD (Hyglos, Bernried am Starnberger Seen, Germany), according to the instructions of the manufacturer. Briefly, three ml of the phage preparations were transferred to a sterile 15 ml Falcon tube (Becton Dickinson) and $CaCl_2$ (Sigma-Aldrich) was added to a final concentration of 0.001 M. Prior to the addition of the phages to the columns, the columns were activated by the addition of 3 ml regeneration buffer. The columns were drained out completely before repeating the addition of the regeneration buffer. Subsequently, 3 ml of the equilibration buffer was added and the columns were drained out completely before a second wash with the equilibration buffer was performed. Finally, the phage samples were added to the column and the eluate was collected in a sterile 15 ml Falcon tube (Becton Dickinson). The columns were then regenerated by washing them twice with 3 ml regeneration buffer. The columns could then be used

for a second purification round or be stored by adding 1 ml of the storage buffer supplemented with 0.02 % sodium monoazide (Sigma-Aldrich). Following endotoxin removal, the titer of the phage solution was determined.

4.2.4.2. Anion-exchange chromatography: Convective Interactive Media (CIM) disk

Phage purification using monolithic anion-exchange chromatography with CIM DEAE disk columns (BIA Separations, Ljubljana, Slovania) was carried out, basically as described by Adriaenssens et al. (2012). Briefly, prior to phage purification, the complete chromatography set up (Äkta FPLC system (GE Healthcare, Little Chalfont, UK with a P900 pump system) was flushed with 1 M NaOH. The loading column was washed three times with endotoxin-free water. Next, approximately 10 ml of one of the high titer phage preparations was loaded onto the chromatography set up. Pump A contained Tris-HCl (20 mM, pH 7.5) buffer whereas pump B contained Tris-HCl (20 mM, pH 7.5) with NaCl (2 M) buffer. Prior to the elution of the phages from the CIM DEAE disk column, the column (containing the phages) was washed with the Tris-HCl (20 mM, pH 7.5) buffer. Elution of the phages from the CIM DEAE disk columns was achieved by washing with an increasing percentage of the Tris-HCl (20 mM, pH 7.5) with NaCl (2 M) buffer relative to the Tris-HCl (20 mM, pH 7.5) buffer.

4.3. Endotoxin quantification

Quantification of the endotoxin concentrations in the differently treated phage preparations was performed using two different commercially available methods. Prior to the endotoxin determination, all phage samples were diluted using Endotoxin-Free Dulbecco's PBS (Millipore, Darmstadt, Germany).

The endotoxin removal efficacy of each purification approach was calculated by first determining the amount of endotoxins per plaque forming unit (pfu) by dividing the endotoxin concentration by the phage titer for each preparation (1).

$$\text{Normalised endotoxin content} = \frac{EU/ml}{pfu/ml} \qquad (1)$$

Subsequently the normalized endotoxin content of each sample was determined by multiplying the endotoxins per pfu with the phage recovery (2), *i.e.* the phage titer of the purified preparation divided by original preparation.

$$\text{Phage recovery} = \frac{(pfu/ml)_{purified\ sample}}{(pfu/ml)_{original\ sample}} \qquad (2)$$

The endotoxin removal efficacy (2) was then calculated by dividing the normalized endotoxin concentration of the purified sample by the normalized endotoxin concentration of the original sample (*i.e.* ϕET or ϕCET) and subtracting this value from 1.

$$\text{Endotoxin removal efficacy} = 1 - \frac{\left[\left({}^{EU/ml}/_{pfu/ml}\right)\times\text{phage recovery}\right]_{purified\ sample}}{\left[\left({}^{EU/ml}/_{pfu/ml}\right)\times\text{phage recovery}\right]_{original\ sample}} \qquad (3)$$

4.3.1. EndoZyme recombinant Factor C (rFC) Assay

According to the manufacturer, EndoZyme (Hyglos, Bernried am Starnberger See, Germany) is an endpoint fluorescent microplate assay intended for *in vitro* quantitative determination of endotoxin (lipopolysaccharides, LPS) in pharmaceuticals and biological substances as well as for medical device testing using recombinant factor C (rFC). The enzymatically active rFC is activated by trace amounts of endotoxins. It is capable of binding both free and bound LPS/lipid A (the biologically potent moiety of LPS) with high affinity. Being at the initial step of the coagulation cascade, Factor C functions as a very sensitive and specific biosensor of endotoxins, capable of detecting pictogram to nanogram levels of endotoxins (Ding and Ho, 2010). Endozyme is able to reliably detect endotoxins within the range of 0.005 to 50 EU/ml. The phage samples were diluted until their endotoxin concentration fell within this range. The assay was performed as described by the manufacturer.

4.3.2. Endosafe-PTS

According to the manufacturer, the Endosafe-PTS (Charles River, CA) is a rapid, point-of-use test system that provides quantitative LAL results within 15 minutes. It is a miniaturized version of the LAL assay using rFC. It is able to detect endotoxins within the range of 0.005 to 10 EU/ml. The endotoxin determination was performed by the VIB Protein Service Facility (Ghent University) as described by the manufacturer.

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Table 1: Different phages used in the different purification strategies. For each phage, taxonomical family and bacterial host strain are presented, as well as the titer of the phage lysates, before application of any endotoxin removal techniques.

Bacteriophage name	Phage family	Bacterial Host	Titer (pfu/ml)	Reference	Isolated by	Isolation date
P. aeruginosa phage PNM	*Podoviridae*	*P. aeruginosa* strain 573	1.8×10^{13}	Merabishvili et al. (2009)	N. Lashki & M. Tediashvili	1999
P. aeruginosa phage LUZ19	*Podoviridae*	*P. aeruginosa* strain 573	5.0×10^{13}	Lammens et al. (2009)	P.J. Ceyssens	2006
P. aeruginosa phage GE-vB_Pae-Kakheti25	*Siphoviridae*	*P. aeruginosa* strain 573	2.5×10^{12}	Karumidze et al. (2012)	N. Kvatadze	2012
P. aeruginosa phage 14-1	*Myoviridae*	*P. aeruginosa* strain 573	3.6×10^{12}	Ceyssens et al. (2009)	V. Krylov	2000
S. aureus phage ISP	*Myoviridae*	*S. aureus* strain ATCC 6538	8.0×10^{13}	Vandersteegen et al. (2011)	Unknown	1920 - 1930

Table 2: Endotoxin removal efficacy. This is defined as the ratio of the endotoxin units (EU) per plaque forming unit (pfu) multiplied by the phage recovery of the purified sample and the original sample substracted from one. Negative values indicate an enrichment of endotoxins. N.D.: not determined. N.A.: not applicable. Dark gray indicates endotoxin removal efficiencies larger than 99%, whereas light gray indicates endotoxin removal efficiencies between 80 – 99 % and white indicates below 80. CsCl: CsCl density centrifugation, OS: organic solvent, TX: Triton X-100, AP: alkaline phosphatase, CIM DEAE: Convective Interactive Media.

Phage name	Lysate (ϕET)	CsCl (ϕC)	CsCl (ϕCET)	(ϕET)	(ϕCET)	(ϕET)	(ϕCET)	(ϕET)	(ϕCET)	(ϕET)
				OS		TX		AP		CIM DEAE
***P. aeruginosa* phage PNM**	N.A.	99.42%	63.09%	99.98%	23.40%	93.21%	56.38%	-9.62%	17.17%	98.15%
***P. aeruginosa* phage LUZ19**	N.A.	18.42%	83.29%	99.68%	-16.55%	N.D.	83.92%	2.84%	20.09%	40.39%
***P. aeruginosa* phage GE-vB_Pae-Kakheti25**	N.A.	99.68%	94.56%	92.97%	63.36%	93.71%	96.95%	-5.83%	22.14%	N.D.
***P. aeruginosa* phage 14-1**	N.A.	69.33%	64.97%	63.83%	63.29%	92.86%	99.69%	-38.96%	7.25%	N.D.
***S. aureus* phage ISP**	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.

Table 3: Titers of single phage after each specific endotoxin removal strategy. All phage titers are expressed as pfu/ml or phage recovery percentage, the amount of phages remaining after the purification as compared to the original sample (phage lysate after Endotrap HD (φET) or CsCl purified phage followed by Endotrap HD purification (φCET), respectively). N.D.: not determined. Dark gray indicates phage recovery above 90 %, light gray between 20 – 90 % and white below 75 %. CsCl: CsCl density centrifugation, OS: organic solvent, TX: Triton X-100, AP: alkaline phosphatase, CIM DEAE: Convective Interactive Media.

Phage name	**Lysate (ϕET)**	**CsCl (ϕC)**	**CsCl (ϕCET)**	**ϕET**	**ϕCET**	**ϕET**	**ϕCET**	**ϕET**	**ϕCET**	**ϕET**
				OS		**TX**		**AP**		**CIM**
	pfu/ml			%						
***P. aeruginosa* phage PNM**	1.80E+13	3.300E+13	3.00E+13	2.06	0.0004	2.00	2.10	2.61	1.57	2.44
***P. aeruginosa* phage LUZ19**	5.00E+13	5.00E+11	5.00E+11	34.00	0.0024	N.D.	14.00	0.02	6.00	0.62
***P. aeruginosa* phage GE-vB_Pae-Kakheti25**	2.50E+12	9.00E+10	9.00E+10	3.20	130.00	17.20	52.22	16.40	8.89	N.D.
***P. aeruginosa* phage 14-1**	3.60E+12	5.00E+12	5.00E+12	26.11	31.00	1.11	1.80	9.44	30.60	N.D.
***S.aureus* phage ISP**	8.00E+13	3.00E+11	3.00E+11	0.03	10.00	0.07	22.80	0.005	0.53	N.D.

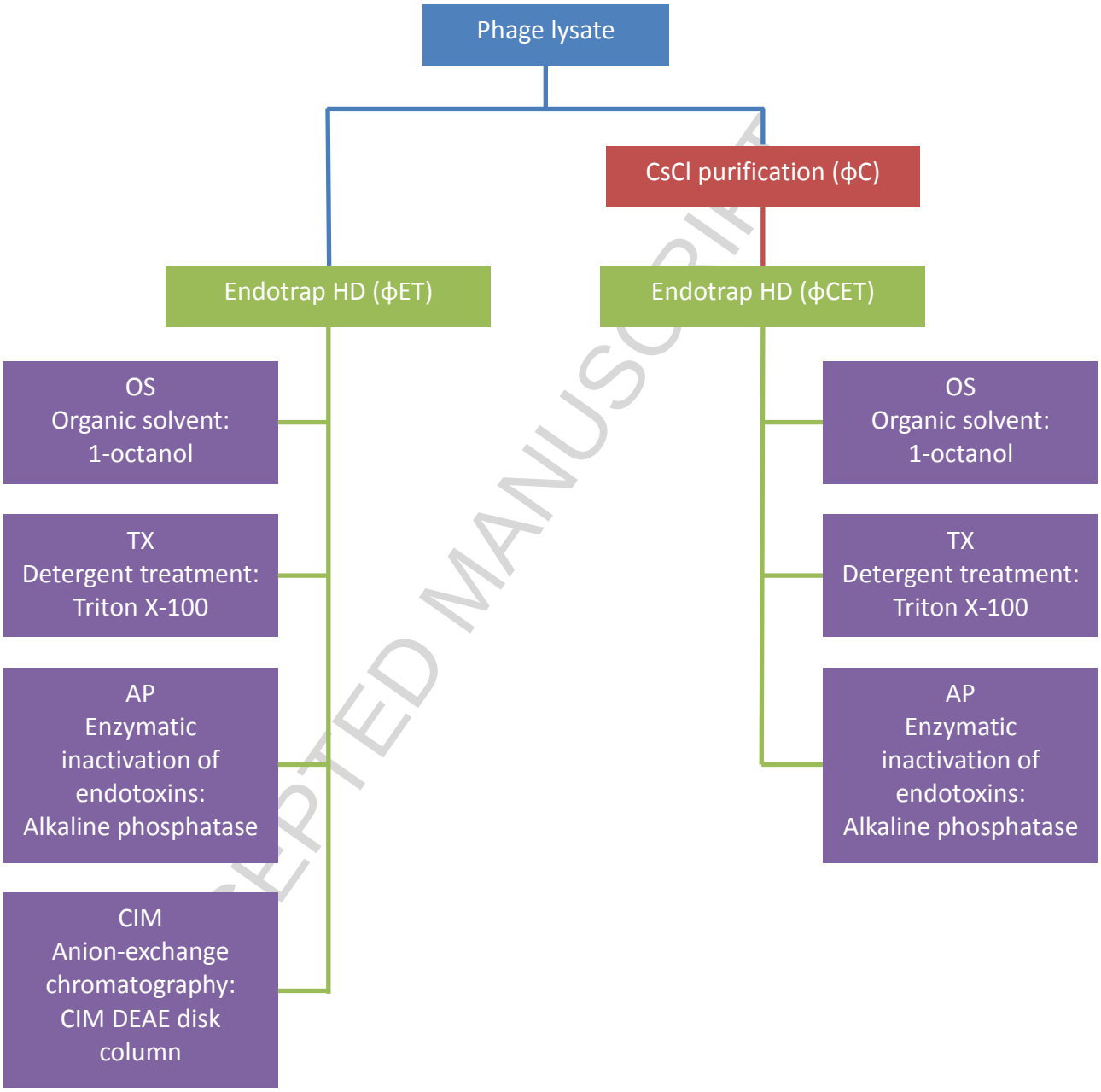

Figure 1: Schematic representation of the different endotoxin strategies used, starting from different phage preparations. Phage lysates were obtained by the overlay-agar method. Part of this phage lysate was used either for **(A)** endotoxin removal using Endotrap HD (φET) or **(B)** further purified through CsCl density centrifugation followed by Endotrap HD (φCET). These preparations were further treated for the removal of endotoxins through different strategies: **(OS)** Organic solvent: 1-octanol; **(TX)** detergent treatment: Triton X-100; **(AP)** Enzymatic inactivation of endotoxins: alkaline phosphatase; or **(CIM)** anion-exchange chromatography: CIM DEAE disk column (only performed on two phages).

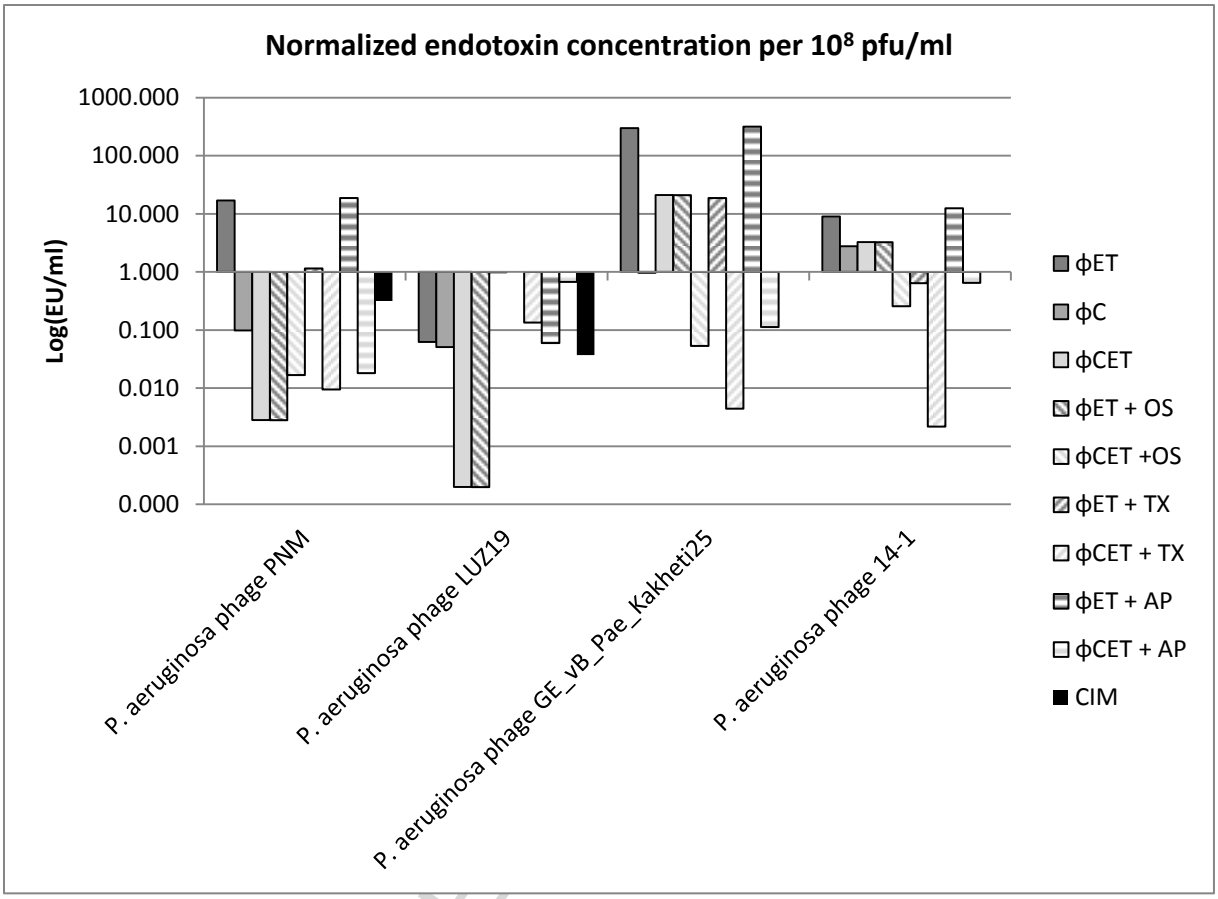

Figure 2: Final endotoxin concentration (EU/ml) present after different purification strategies for four different *P. aeruginosa* phages, normalized against a phage therapeutic titer (*i.e.* 10^8 pfu/ml). φET: lysates after Endotrap HD, φC: CsCl purification, φCET: φC after Endotrap, OS: Organic Solvent, TX: Triton X-100, AP: Alkaline Phosphatase, CIM DEAE: Anion Exchange using CIM DEAE disks.

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Highlights

- The endotoxin removal efficacy is phage specific for the different tested strategies.
- The endotoxin removal strategies affect phage infectivity to different degrees.
- CsCl density centrifugation leads to the highest endotoxin removal efficacy of the tested endotoxin removal strategies, with a reduction up to 99 %.
- Complete removal of endotoxins was not obtained, a minimum of 2.84 % could be removed, leaving a maximum of 7,000,000 EU/ml in the phage preparation.